NASA microgravity research highlights

Looking to the Crystal for Answers

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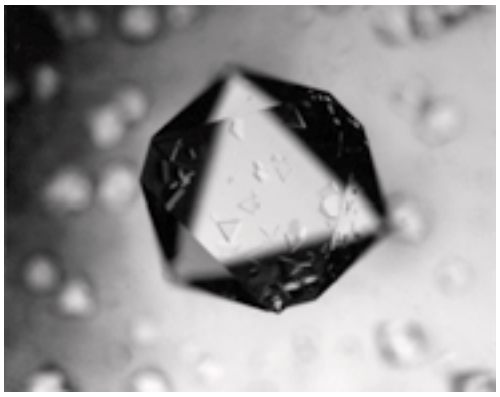
Like its namesake crystals, NASA's protein crystal growth program has burgeoned from a small nucleus to a well-developed, multifaceted entity, with its components all headed the same direction and toward the same goal — reaching a better understanding of growing crystals and solving the structure of proteins that are key to understanding biological functions. This final article in the review of the Microgravity Research Program disciplines looks at the protein crystal growth program from its beginning only 15 years ago, through its expansion into pharmaceutical applications, to its future as the completion of the International Space Station draws nigh.



This unusually large cubic crystal of satellite tobacco mosaic virus grown on the second International Microgravity Laboratory (IML-2) mission is more than 30 times the size of similar crystals grown on Earth.

Lifetimes of hours, millions of dollars, and mountains of hope have been invested in attaining some of the world's tiniest crystals — crystals that, when nearly perfect in form, are some of the most highly prized today. Yet the crystals — protein crystals — have no value in and of themselves. What makes them so sought after is the information they reveal about a protein's molecular structure. Alexander McPherson, a principal investigator for NASA in protein crystal growth at the University of California, Irvine, explains, "Once you've gotten those data from the crystal, you can throw the crystal away. The crystal itself has no material value at all. None."

So what puzzles are McPherson and other researchers trying to solve with data provided by protein crystals? Proteins — macromolecules involved in everyday functions of the body such as transporting oxygen and chemicals in the blood, forming major components of muscle and skin, and fighting disease — come in over 100,000 varieties. Active sites on some of the proteins, when inappropriately triggered, can cause disease or an unwanted function. Scientists need to locate those active sites so drug designers can understand the sites and then work to block them or render them inactive. Craig Kundrot, a researcher at Marshall Space Flight Center, explains, "The ibuprofen that you take after that busy day of gardening works on a specific protein [involved] early in the signaling process that tells the rest of your body, 'We should have some inflammation here.'" Blocking the active site on the protein prevents or alleviates the inflammation.

The trouble is, proteins are so small that humans can't see them individually, let alone find a specific site on one or determine its atomic structure. Kundrot illustrates, "If one cell in my body were the size of the Houston Astrodome, one protein molecule would be the size of a can of Coke, and one atom would be the size of the fine print on the can."

To better see these key regulators of the human body, researchers grow crystals. A protein crystal is a three-dimensional array of molecules in which each molecule has the same orientation in the same chemical environment and has the same relationship to its neighbors. The effect of bringing these molecules together in this arrangement is amplification. As McPherson explains, "Think of it as a football stadium full of people. If only one person stands up and yells, you don't hear very much. But if everybody stands up, faces the same direction, and yells at the same time, you can hear that a long way away. That's exactly what happens in a crystal. One molecule by itself would give you a signal that would be so weak that you wouldn't ever be able to detect it. But because all the molecules in the crystal are doing exactly the same thing at exactly the same time, we can record the signal, and we can figure out what the signal from a single molecule is." The better ordered the molecules are in the crystal, the better the signal, and the better the information for drug designers.

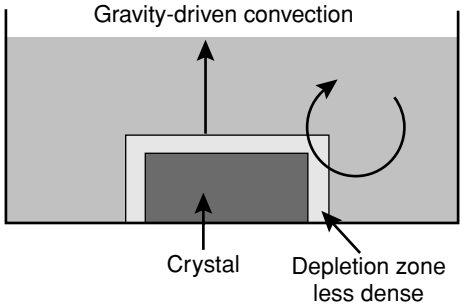
150 Years on the Ground

The first known published observation of the crystallization of a protein was made by F. L. Hünefeld at Leipzig University in Germany. While working with hemoglobin in 1840, Hünefeld obtained flat, plate-like crystals of this iron-containing protein when he pressed the blood of an earthworm between two slides of glass and allowed the blood to dry very slowly. In 1851, Otto Fünke, another German researcher, published a series of articles in which he described growing crystals by successively diluting blood corpuscles with pure water or alcohol or ether, followed by slow evaporation of the protein solution.

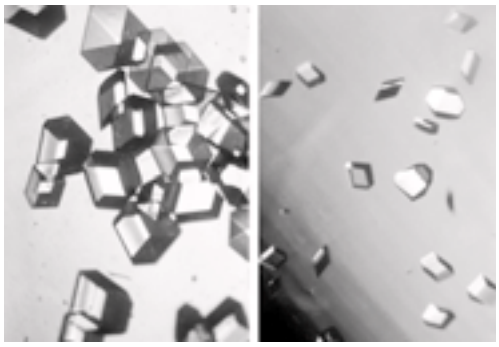
Early on, scientists grew crystals solely to purify the proteins. Not until the 1930s did researchers begin to focus their attention on crystals as a source of structural information about molecules. They turned to X-ray diffraction, a procedure using a pencil-lead-sized X-ray beam directed at the crystal. The X-ray beam is scattered by the crystal into tiny points of light recorded on a film, helping to reveal the molecule's structure. By the 1960s, X-ray diffractionists were investigating an abundance of crystals grown by biochemists. In addition, there was a century-long backlog of protein crystals to be investigated. By the 1970s, however, the diffractionists had become selective regarding their crystals, and supplies were no longer meeting demands. New methods for growing higher-quality crystals were needed.

Crafting Crystals Among the Stars

During that decade, Walter Littke, a space research pioneer and professor of chemistry at the University of Freiburg, Germany, was using a common method of growing crystals, placing a salt solution together with the protein solution. When the two solutions came in contact, the salt began affecting the protein's sol-



Gravity or density-driven convection occurs as protein molecules incorporate into a crystal lattice from the surrounding solution. The layer bordering the crystal (the depletion zone) then contains a less-dense protein concentration, causing the layer to rise. The remaining, denser solution sinks because of gravity, creating eddies that make it difficult for more protein molecules to attach to the crystal in an ordered way.



Larger, better-structured insulin crystals grown in microgravity (left) reveal their vital form and function to crystallographers and to scientists seeking better treatments for diabetic patients. The same information would be almost impossible to obtain from the smaller, less well-ordered insulin crystals grown on Earth (right).

vent, concentrating the protein solution until the protein began to crystallize. The resulting crystals, however, were fragile, and small or broken.

By 1980, Littke suspected the culprit in his unusable crystals to be density-driven convection, or fluid flow, a phenomenon that occurs in normal gravity. Density-driven convection takes place during crystal growth as protein molecules diffuse from the surrounding solution and add in an orderly way to the growing crystal lattice. The solution bordering the crystal then contains a lower protein concentration than the remainder of the solution and, therefore, a lower density. This less-dense solution tends to rise, and the denser solution sinks under the influence of gravity, creating eddies next to the crystal. These convective currents are harmful because they can alter the orientation and position of the protein molecules as they add to the crystal lattice, thereby causing disorder of the lattice. This affects the resolution, or clarity, with which a crystallographer can "see" the precise position each atom occupies in the three-dimensional structure of the protein.

Another adverse effect of gravity on growing crystals is sedimentation. Crystals drift to the bottom of a drop of the solution when they have grown to a mass larger than can be supported by suspension in the drop. When this happens, partially formed crystals may fall on top of one another and continue growing into each other. Since X-ray diffraction analysis requires single crystals, sedimentation renders potentially high-quality crystals unusable for data collection.

To test his ideas about these gravity-related problems, Littke sent a protein crystal growth experiment up on a British sounding rocket, theorizing that in microgravity, the gentle movement of molecules by diffusion alone (as protein molecules randomly drift from areas of higher density to areas of lower density) would allow better crystals to grow. Video of the experiment, which lasted only about 6 minutes, showed evidence that crystal growth in microgravity was indeed quite different from the same process on Earth.

With NASA support, Littke followed up by sending beta-galactosidase (a bacterial enzyme important in sugar metabolism) and lysozyme (a protein found in egg whites that is useful for modeling the crystallization process) on the space shuttle. When the shuttle landed, Littke found lysozyme crystals larger than those previously produced by the same method on Earth. The crystals of beta-galactosidase were significantly larger and of fairly good quality. This was even stronger evidence that growing crystals in microgravity could have a profound effect on the final product.

Meanwhile, across the Atlantic, NASA scientists from Marshall Space Flight Center (MSFC) were laying the groundwork for further protein crystal growth in microgravity at a joint gathering of researchers at the University of Alabama, Birmingham (UAB). Charles Bugg, a professor of biochemistry at UAB Medical School and associate director of the school's Comprehensive Cancer Center, attended that meeting and was excited about the possibilities presented. He discussed some of the ideas with Lawrence DeLucas, who is the current director of the Center for Macromolecular Crystallography at UAB, a NASA Commercial Space Center, and was then a faculty member at UAB. DeLucas remembers, "When Bugg came to me and said, 'Hey, have you heard about growing crystals in space?', like most people the first time they hear it, I laughed. And I said, 'Why in the world would we want to do that?'" But DeLucas decided to listen to the presentation at the next joint gathering. "So I went there and my eyes opened up. I saw all kinds of possibilities to improve our ability to grow crystals. Then I started talking to NASA and asking, 'How do you do this?'"

DeLucas agreed with Littke's suspicions about the detrimental effects of gravity on growing protein crystals. He believed growing the crystals in microgravity would result in bigger and more highly ordered crystals, which would provide better, clearer information for reading their structures. Proof of all of these theories began to materialize as protein crystal growers sent experiments into space.

The first protein crystal to be grown in space using vapor diffusion, a common crystal growth method, was part of an experiment by Bugg and DeLucas in 1985. They adapted the method for use in microgravity so they could test a broad array of proteins in a manner similar to how they were tested on Earth. The two investigators launched almost 50 vials of 10 various proteins on shuttle flight STS-51D. The one successful crystal came from a lysozyme solution and was 10 times the size of any other lysozyme crystal grown using any method on Earth.

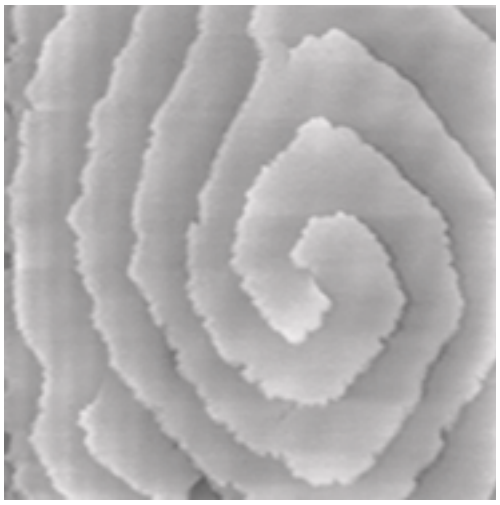
To better understand the results from the protein crystallization experiments conducted in microgravity, NASA started a research program on protein crystal growth in 1991. The goal of the new program, led by MSFC, was to answer some of the central questions about growing protein crystals: How does fluid flow affect crystal growth? How might microgravity reduce nucleation, which on Earth tends to happen too quickly and produce too many tiny crystals instead of a few large ones? What new approaches to protein crystallization in microgravity would improve the flight results or increase the number of samples flown?

Daniel Carter, formerly chief scientist for biophysics at MSFC and now founder and CEO of New Century Pharmaceuticals Inc. in Huntsville, Alabama, helped address some of these questions with the Protein Crystallization Apparatus for Microgravity, or PCAM, which he developed. The PCAM has a much higher capacity for microgravity protein crystallization experiments and allows easier loading of samples before a mission. It flew its first flight in 1995. Carter recounts one of the numerous crystallization successes that followed the first PCAM flight. "We were collaborating with professor Mark Wardell, originally at Cambridge University, now at Washington University in St. Louis, Missouri, who had been working on the structure of human antithrombin II, a major protein involved in blood clot formation. Wardell and his research group had been studying the protein since 1991 but had not been able to resolve important details in the active site of the molecule, particularly a large flexible loop that was presumably disordered. But when they grew these crystals in microgravity in 1995, the crystals were of much higher quality, allowing them to finish the structure and visualize important details in the active site for the first time."

In this case and in many others, there is a strong tie between conducting an experiment in microgravity and achieving better-ordered and much larger crystals. Carter expands, "If you look at all the examples, there's a strong correlation. People were comparing these to the best results they've had on the ground over a period of years. As far as we know [for these cases], the high resolution is achievable only through microgravity."

Blueprint for Growing a Crystal

But just how do scientists grow a crystal in microgravity (or on Earth, for that matter), and what do they do with it once they have it? Researchers begin with a protein solution that is supersaturated. Proteins (like many molecules, such as salt) have a solubility, which is the amount of the molecule that can dissolve in



Atomic force microscopy uses laser technology to reveal a defect, a double-screw dislocation, on the surface of this crystal of canavalin, a major source of dietary protein for humans and domestic animals.

a given solvent. Growing a crystal, however, requires supersaturation, which is a thermodynamically unstable solution with more than the nominal amount of protein required for solubility.

The trouble is, most scientists don't know the solubility of their protein. "Most protein crystals have been grown by trial and error, using some sort of screening method. A researcher tests many, many different solvent conditions in hopes of finding one that the protein likes enough to form a crystal," says William Wilson, associate director of research at the Center for Macromolecular Crystallography and a professor of chemistry at Mississippi State University. This gap in knowledge adds difficulty to the process of growing crystals, so Wilson has devised a means of measuring the dilute solution property of a protein solution to predict which solution conditions are apt to be favorable for crystallization. He shines a laser beam through a protein solution and measures information from the scattered light to quantify the solution's behavior. The result is called a second virial coefficient, which, in effect, tells how the protein molecules are interacting with each other. Positive values for the coefficient indicate repulsion between protein molecules while negative values mean that protein molecules are attracted to each other. Values of the coefficient that turn out to be good for protein crystallization are slightly negative and define the "crystallization slot."

Once the researcher has a workable solution, he or she usually uses one of two methods for actually growing the crystal, vapor diffusion or liquid/liquid diffusion. Both methods involve equilibrating a protein solution against a precipitant. In vapor diffusion, the method used in the PCAM, the crystal grower evaporates a drop of protein solution a little bit to make it more concentrated, and then waits for a crystal to form. In liquid/liquid diffusion, the researcher diffuses a salt solution or some other precipitant from one compartment of a two-chamber vial into a protein solution from the other compartment, and as the salt concentration increases, the water from the protein solution is drawn out, the concentration of the protein in the solution rises, and the protein begins to crystallize.

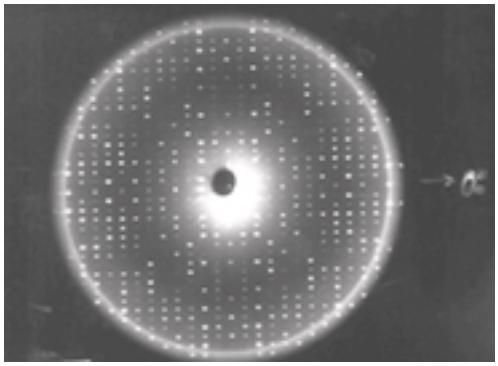
McPherson explains how crystals form once nucleation has begun: "Ideally, molecules of protein (or DNA, RNA, or viruses) organize themselves so that every molecule has identically the same orientation in space and identically the same chemical environment throughout the crystal. In other words, we have millions upon billions upon billions of absolutely identical molecules all facing the same direction, all having exactly the same relationship to their neighbors, and it's what we call a periodic array or an ordered [three-dimensional] array of molecules."

When a crystal grows, attachment kinetics and transport kinetics are competing for control of the molecules. As a molecule gets close to the crystal surface, it has to attach properly for the crystal to be usable. NASA has funded investigators to look at those attachment kinetics from a theoretical standpoint and an experimental standpoint. McPherson is one of those investigators. He uses X-ray diffraction and atomic force microscopy in his laboratory to answer some of the many questions about how protein crystals grow. Atomic force microscopy provides a means of looking at how individual molecules are added to the surface of growing protein crystals. This helps McPherson understand the kinetics of protein crystal growth. McPherson asks, "How fast do crystals grow? What are the forces involved? Investigators funded by NASA have clearly shown that such factors as the level of supersaturation and the rate of growth all affect the habit [characteristic arrangement of facets] of the crystal and the defects that occur in the crystal."

Choosing a protein crystal to read for habit, defects, and structure has been limited to looking at size and outward appearance, a time-consuming process that also can give only limited information. Recently, however, Wilson developed a new method to give protein crystals a "score." He uses a fluorescence microscope to look at protein crystals in any container they're grown in. He explains, "We can look at the individual crystals and do a very quick analysis of the fluorescence from a particular crystal, and analyze that fluorescent signal and make a judgment, then, as to whether that one is worthwhile doing the [costly] X-ray crystallography on."

To analyze the selected crystal, an X-ray crystallographer shines X-rays through the crystal. Unlike a single dental X-ray, which produces a shadow image of a tooth, these X-rays have to be taken many times from different angles to produce a pattern from the scattered light, a map of the intensity of the X-rays after they diffract through the crystal. The X-rays actually bounce off the electron clouds that form the outer structure of each atom. A flawed crystal will yield a blurry pattern; a well-ordered protein crystal yields a series of sharp diffraction patterns.

From these patterns, researchers build an electron density map. With powerful computers and a lot of calculations, scientists can use the electron density patterns to determine the structure of the protein and make a computer-generated model of the structure. The models

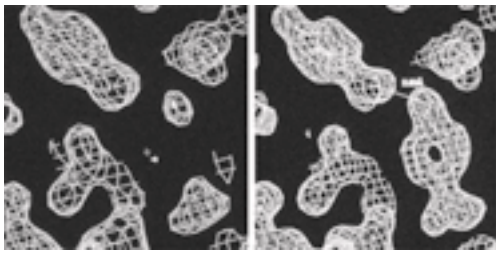


X-rays diffracted from a well-ordered protein crystal create sharp patterns of scattered light on film. A computer can use these patterns to generate a model of a protein molecule.

let researchers improve their understanding of how the protein functions. They also allow scientists to look for receptor sites and active areas that control a protein's function and role in the progress of diseases. From there, pharmaceutical researchers can design molecules that fit the active site, much like a key and lock, so that the protein is locked without affecting the rest of the body. This is called structure-based drug design.

Going From Proteins to Pharmaceuticals

Wilson asserts that, in the end, all of the protein crystal growth technology developed through NASA-sponsored research will "help pharmaceutical companies to gain better models of proteins that are related to certain diseases like diabetes. Once those protein structures are determined, that will allow the structure-based design community to design their drugs." DeLucas avers, "One of the most exciting areas of protein research has been insulin and the work the Hauptman-Woodward Medical Research Institute in Buffalo, New York, is doing on diabetes. Although the structure of insulin was done long before we grew space crystals, what we're flying is insulin with drugs designed by investigators at the Hauptman-



These sharp, complex blueprints of insulin molecular structures are based on crystals grown in microgravity (left). Computer-generated insulin models from crystals grown on the ground (right) omit a lot of information that is key to unlocking the mystery of how insulin works.

Woodward Institute soaked into the crystal or co-crystallized with it. The ultimate goal is perhaps a longer-acting insulin formulation, also a faster-acting formulation. If you've just had a meal, you need something that acts quickly. If you wake up in the morning, you'll want something that has a slow basal release of insulin over a long period of time, and today many diabetics every morning inject themselves with this slow-acting formulation. If you could come up with a formulation so they don't inject themselves but once every three days, that improves the quality of their life dramatically, and it may help with the long-term complications by giving them a more steady release of the insulin. Another one is the protein factor D, a part of our complement system, which plays a destructive role when there are complications from open heart surgery. Flying factor D in space helped us determine its structure, and the project has proceeded to where today there are drugs in clinical trials."

Kundrot is working with the protein that makes staphylococcus A drug-resistant to the penicillin class of antibiotics. "This is the worst infection you can pick up in the hospital. The protein has not been crystallized before, but we're going to give it a go and see if microgravity can help us out on that." Kundrot says his laboratory is "very keen to work on problems that other people have given up on, especially pharmaceutical targets, such as the penicillin binding protein from staph A."

In fact, microgravity has been the chosen environment for dozens of pharmaceutical and fundamental experiments growing macromolecules. Since NASA's protein crystal growth program began, PIs and their research teams have flown samples of a total of 185 different proteins, RNAs, DNAs, and viruses. Over the years, those teams have grown from the 3 co-investigators and 5 guest investigators who worked with Bugg and DeLucas on their first flight experiment to the current level of 20 co-investigators and 63 guest investigators. The proteins, RNAs, DNAs, and viruses these scientists study range from insulin to lactate dehydrogenase (a major enzyme in energy production and an extremely important muscle protein in all animals) to thaumatin (a sweet-tasting protein with potential as a sugar substitute). Most of NASA's protein crystal growth experiments, conducted from 1985 to 1999, have been flown on space shuttle missions. The remainder were conducted on Russian Space Station Mir. Among the experiments have been NASA-sponsored research as well as experiments from pharmaceutical companies that have needed better crystals than what they could grow on Earth. In the latter cases, the company bears the cost of producing the protein solution, taking the solution to the launch site, and returning to the site to pick up the crystals after the flight. NASA provides the hardware and flies the experiment for the company, which analyzes the crystals and then supplies NASA with information regarding whether or not the crystal was better. All other data about the crystals is proprietary and remains solely with the company.

What Future Do the Crystals Hold?

Given the great strides that NASA's protein crystal growth program has made over the past decade and a half since the first protein crystal growth experiments were conducted in microgravity, where is the field headed now? For many, it's to the International Space Station (ISS).

The ISS will expand the opportunities for growing crystals in microgravity, enabling continued advances in developing new pharmaceuticals and understanding the growth process of crystals. Crystals, which usually grow more slowly in microgravity, will have time to fully develop into usable specimens without disturbance from thrusters, which fire periodically on shuttle flights to correct the attitude of the craft and which can disrupt or alter the growth of potentially well-ordered crystals. Neither will crystals disintegrate because of lack of temperature control, as was sometimes the case on Mir. And finally, follow-up experiments, an important and common feature of ground-based research, will be more feasible in space as researchers "send up protein solutions, gain access to progress of the crystal growth through telescience [or some other means], and analyze which parameters need to be adjusted for better crystals," explains Kundrot.

New facilities for growing crystals on the ISS are in the works. The GN2 Dewar, developed by McPherson, may be used routinely on the ISS. The dewar is designed primarily to grow large numbers of different proteins, nucleic acids, and virus crystals under lots of different conditions on a single space mission. A NASA-funded colleague of McPherson's at the University of California, Irvine, Alexander Malkin, hopes to send atomic force microscopy, a technique that's only recently (since 1992) been used to study the surface of macromolecular crystals, up to the ISS. Malkin explains the benefit of sending this technology to space: "We can show in-situ, in controlled conditions, the changes in crystal growth [as different parameters are adjusted] under microgravity conditions so we can understand what actually happens."

Another microgravity application just beginning to be explored by Carter and his research group involves the production of extremely large protein crystals for application in neutron diffraction. Carter explains, "Neutrons interact with the nucleus of the atom instead of the diffuse electron clouds, allowing us to see things like the precise location of hydrogen atoms in water molecules and within the active sites of proteins. We have recently completed our first structure. It's very exciting."

With all of these new technologies ready or being prepared for use on the ISS, DeLucas hopes the spacecraft will include a full protein crystal growth laboratory, allowing all kinds of procedures, from mixing protein solutions to reading fully grown crystals. His team has already built a prototype X-ray generator that works on only 25 watts instead of 6 kilowatts (the energy required to power most generators on Earth), as well as a robotic system to mount the crystals. All of these current or fast-approaching advances in flight and ground research evidence the benefits that continued NASA support brings to the field of protein crystal growth in general and structure-based drug design in particular. Partnership with various institutes and pharmaceutical companies is advancing research with crystals of proteins such as malic enzyme, a key protein in the life cycle of intestinal parasites; gamma-interferon, which stimulates the body's immune system and is used clinically in the treatment of cancer; and porcine elastase, an enzyme useful in studying causes of emphysema. NASA's continued teamwork with universities and with pharmaceutical companies will ensure that more and more secrets the tiny crystals hold will be unlocked in microgravity for the advancement of human health. DeLucas says, "That unique environment makes us think differently than we would without going to it and understanding how it affects these [protein crystal growth] processes."

Additional information

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